COLLAGEN — A NATURAL SCAFFOLD FOR BIOLOGY AND ENGINEERING

by

ELEANOR M. BROWN*

U. S. Department of Agriculture**, Agricultural Research Service
Eastern Regional Research Center,
600 E. MERMAID LANE,
WYNDMOOR, PENNSYLVANIA 19038

ABSTRACT

Collagen, the most abundant protein in mammals, constitutes a quarter of the animal's total weight. The unique structure of fibrous collagens, a long triple helix that further associates into fibers, provides an insoluble scaffold that gives strength and form to the skin, tendons, bones, cornea and teeth. The ready availability, to meat eaters, of animal skins that would putrefy, if left untreated, led to man's earliest venture into biomaterials engineering and resulted in the production of leather. Through empirical methods, a number of tanning agents with a variety of properties were identified. The methods for production of leather evolved over several centuries as art and engineering with little understanding of the underlying science. Scientific advances of the twentieth century, including increasing use of collagen in medical biomaterial research, began to provide a basis for understanding the relationship between collagen structure and function in both biology and technology.

During the past 20 years, leather researchers at ERRC have used experimental and theoretical approaches to investigate several methods for stabilizing collagen structure. This research, which includes studies of mineral and vegetable tannages, enzyme-catalyzed and aldehyde-based covalent crosslinks, electrostatic and hydrophobic interactions, will be reviewed. Insight gained from these studies and those of other leather and biomaterials scientists will be evaluated as steps toward a still elusive, comprehensive mechanism for stabilization of collagen in leather and other biomaterials.

RESUMEN

El colágeno, la proteína más abundante en los mamíferos, constituye una cuarta parte del peso total del animal. La incomparable estructura fibrosa del colágeno, una larga triple hélice que luego se asociará en fibras, proporciona un andamiaje insoluble que le da fuerza y forma a la piel, tendones, huesos, córnea y dientes. La disponibilidad, a consumidores de carne, de pieles de animales que se pudren si se dejan sin tratamiento, llevó a los primeros hombres a la ingeniería de biomateriales dando como resultado la producción de cuero. A través de métodos empíricos, una serie de agentes curtientes con una variedad de propiedades fueron identificados. Los métodos para la producción de cuero evolucionaron a lo largo de varios siglos como arte e ingeniería con escasa comprensión de la ciencia subyacente. Los avances científicos del siglo XX, incluido el aumento de la utilización del colágeno en la investigación de dispositivos médicos, comenzó a proporcionar una base para entender la relación entre estructura y función del colágeno en biología y en tecnología.

Durante los últimos 20 años, los investigadores del cuero en ERRC han utilizado los enfoques teóricos y experimentales para investigar varios métodos para la estabilización de la estructura de colágeno. Esta investigación, que incluye el estudio de curtidos minerales y vegetales, enzimas catalizadoras de reticulación aldehídica covalente y basada en aldehídos, interacciones hidrófobas y electrostáticas, serán reevaluadas. Los conocimientos adquiridos a partir de estos estudios y los de otros estudios científicos sobre cueros y biomateriales serán evaluados como pasos iniciales para esclarecer un todavía elusivo mecanismo comprensivo de la estabilización del colágeno en la piel y otros biomateriales.

INTRODUCTION

It is a great honor to be invited to present a John Arthur Wilson Memorial Lecture. This year's lecture is the 50th in the series. As a relative new comer to leather science, I am most humbled by the stature of the previous 49 lecturers, most of whom dedicated their entire careers to this complex and fascinating field. I thank the 2009 Wilson lecture committee for selecting me, and Stahl USA for their generosity in support of this lecture series.

Previous lectures in this series have covered a wide range of topics from basic science of collagen to physical properties of leather products, environmental issues and economic considerations. Most speakers have used the opportunity to summarize their own research in a historical context, looking both back and forward. A major theme in my research is the relation between protein structure and protein function in biology and technology. A continuing commitment of mine is to the ALCA Committee on Uses of Collagen and Its Coproducts. In this talk, I plan to emphasize these topics of interest, highlight earlier work by ERRC scientists in these areas, and look at some of the newer uses of collagen that may eventually impact the tanner's approach to this valuable resource. The talk is organized in three parts: Early Processes and Models, Leather Models, and Biomaterial Models.

Early Processes and Models

The shape and mechanical attributes of a vertebrate body are defined by its connective tissue. The cells of connective tissue are embedded in an extracellular matrix that is a complex mixture of proteins and carbohydrates and functions as a support for cellular materials. Collagen, the primary protein of the extracellular matrix, is the most abundant protein in mammals, and has throughout history played important roles in physiology and technology. Scientific advances of the twentieth century, including increasing use of collagen in medical biomaterial research, began to provide a basis for understanding the relationship between collagen structure and function in both biology and technology.

From the days of the hunter/gatherer, it was recognized that those parts of an animal that remained after the meat was consumed could be put to other uses. Animal skins served as protection against the elements and dried bones as useful tools. The role of collagen as a technologically important biopolymer dates to prehistory. Dried bones from carcasses of animals that died naturally or were killed by man or other animals were shaped to form awls and projectile points, at least as far back as the Middle Stone Age, as evidenced by bone tools recovered from a 70,000 year old site in South Africa.[1]

The earliest known example of processed collagen in the form of glue is in the cave paintings in Lascaux, France.

Artifacts from Egyptian tombs show the use of glues in the manufacturing of furniture veneers and in the production of papyrus. Gelatin was most likely discovered when boiled bones leached material that gelled when cooled. By the Renaissance period, chefs took pride in their gelatin molds, and in the 19th century, dinner party tables were graced with elaborate castles and other constructions made of gelatin. The makers of sausage, perhaps the first convenience food, employed the intestinal tracts of animals (largely collagen) to contain and protect their product.

Medical use of collagen was another early development as evidenced by the description of the catgut sutures in surgery in ancient Egypt.[2] By the middle of the 19th century, the practice of stabilizing collagen structure by soaking the sutures in a solution of trivalent chromium had been developed as well as the use of glycerol to make the sutures more pliable.

Leather has always been a byproduct industry. The ready availability, to meat eaters, of animal skins that would putrefy, if left untreated, led to another advance in biomaterials engineering that resulted in the production of leather. Soaking and stretching dried hides in animal fat or brain tissue produced the earliest leathers. Treatment with adipose and other fatty tissues helped to soften the hide while giving it a coating that protected against damage by water and microbes. Vegetable tanning, in which stacks of hides in pits were soaked with tree bark, often from oak trees, was well established by the Middle Ages. Early vegetable tanning took many months because the tree bark tannins were weak, and diffusion through a stack of hides was slow. The advent of mixed tannin and spray-dried extracts, synthetic polyphenols, and the use of drums instead of pits shortened the process to a few days for production of specialty leathers, used in saddles, harnesses, belts, and shoe soles and a variety of crafts.[3] Over time, through empirical methods, a number of tanning agents that produced leathers with a variety of properties were identified. The methods for production of leather evolved over several centuries as art and engineering with little understanding of the underlying science. By the 1880's, tanners had adopted chrome tanning, based initially on the process developed early for stabilizing sutures by soaking them in a Cr (III) solution.

Technical advances of the twentieth century, including the increasing use of collagen in medical biomaterials, began to provide a basis for understanding the relationship between collagen structure and function in both biology and technology. The early 20th century was a time of rapid development in both basic sciences and applied technologies. The academic scientists and the technologists (tanners) had little contact with each other, and essentially spoke different languages. In general, each group failed to appreciate and benefit from the other's work. John Arthur Wilson, in whose

memory this lecture series was established, spent his lifetime merging the practice of tanning with the academic science of collagen. He was a master of the theoretical and the practical aspects and had both the opportunity and ability to combine these in the development of a basis for new technologies. He was an active member of our Association, a frequent contributor to JALCA, and served as an interpreter of basic science to the industrial leather chemists and technologists. Three of his publications were major reviews of the then current knowledge of the relationship between collagen function and structure.

Wilson's lecture at the 1919 ALCA meeting was entitled "The application of colloid chemistry to the leather industry."[4] Colloid chemistry was then defined as "the chemistry of reactions occurring at or near the surface in a system where surface area was large relative to the mass of material." Tanning was thought to occur primarily through electrostatic interactions between the tannin molecules and charged sites on the surface of the animal skin. The following decade was one of rapid advances in most scientific areas, and leather science more or less kept pace with other scientific developments.

Nine years later, in the 1928 Chandler Lecture at Columbia University, entitled "Chemistry and Leather," Wilson recognized that leather chemistry was very much concerned with the molecular structure of the protein, collagen.[5] Proteins were known to be composed of amino acids, some with known structures, but little was known about the arrangement of the amino acids within the protein. Questions concerning the size and structure of individual proteins and macromolecular protein complexes were yet to be addressed. Some of the issues that Wilson dealt with in that lecture are with us still, and illustrate the complexity of collagen/tanning agent interactions. He realized that the interaction between collagen and chromium was not the simple binding of a Cr^{+3} ion to collagen, but he lacked the detailed structures of collagen and chromium coordination complexes necessary to propose a more comprehensive mechanism for chrome tanning. Similarly, the structural chemistry of vegetable tannins was in its infancy.

Wilson's final JALCA publication, the 1943 paper, "Protein Structure and the Mechanism of Tanning," coauthored with Porth,[6] was based on his presentation at the 1941 ALCA meeting. The collagen structure of skin was then viewed as winding layers of polypeptide chains stabilized by hydrogen bonds. Structures were beginning to be proposed for vegetable tannin molecules, and those with the potential for multipoint fixation to collagen were seen as true tanning materials. The effect of a tanning material on the shrinkage temperature was recognized as a reliable indicator of degree of tanning.

Scientists at the Eastern Regional Research Center, USDA (ERRC) have long performed research in the area between the basic science of the academy and the applied technology of an industrial setting, with the aim of balancing basic and applied approaches to each research problem. Much of their research has ultimately been incorporated into industrial processes. Because the adoption of research results by industry is typically a rather long process, and the origin of the work may be clouded by the time the concept is widely applied, I have chosen a few accomplishments of ERRC leather researchers between 1945 and 1990 to illustrate research on collagen structure and tanning mechanisms that has impacted the industry in process development and understanding.

In leather processing research of the 1950's and 60's, ERRC scientists evaluated the tanning potential of dialdehyde starch,[7] and developed processes for glutaraldehyde tanning of shearlings to produce washable bed pads for hospital use,[8,9] research that provided the basis for today's chrome-free leathers. Chromium-glutaraldehyde combination tannage was reported in the 1970's.[10] During the 1970's and 80's, considerable research effort was directed toward the science behind approaches to solving environmental issues of the tannery, such as treatment of beamhouse effluent[11] and fleshing machine offal.[12]

In fundamental studies of collagen structure, ERRC leather scientists of the 1940's and 50's used electron microscopy, a then emerging technique, to obtain micrographs of cattle hide collagen in its native state[13] and after the various steps of beamhouse processing.[14] They also studied the properties of soluble and insoluble collagen under a variety of conditions with the aim of finding new uses for collagen as a texturizer in the food industry.[15] ERRC scientists of the 1970's examined the effects of hydration on collagen structure[16] and the flexibility of the nonhelical regions.[17] The fundamental understanding of collagen structure was advanced in the 1980's through studies of fibril assembly.[18,19]

By 1990, after half a century of studies, collagen had been defined as a family of proteins located in the extracellular matrix of connective tissue that provides a structural basis for the mechanical and biochemical properties of tissues and organs.[20] Fibrous components of connective tissue were first observed in the 19th century, and by the 1920's, the connection between gelatin and the fibrous structure of connective tissue had become apparent.[21] Several distinct types of collagen had been identified, which collectively, represent about one third of the total protein of vertebrate animals. As a function of structure and supramolecular organization, they were grouped as fibril-forming (types I, II, III, V, XI), fibril-associated (types IX, XII, XIV), membrane (types IV, VII, VIII, X) or other specific function. The fibril-forming collagens are the major structural element of

connective tissue, providing the scaffold that gives stability and integrity to tissues and organs. The primary collagen unit, tropocollagen, is a triple helix comprised of three separate polypeptide chains. Fibrillar collagens are triple-helical over essentially their entire length. In addition to physiological function, where they give structure to tissues and organs, the fibril-forming collagens are technologically important as molecular frameworks in the leather, food and medical industries.[22]

Collagens are noted for their high content of glycine (Gly), proline (Pro) and hydroxyproline (Hpr). The primary structure of the helical domain of collagen is the repeating tripeptide $(Gly-Xxx-Yyy)_n$. Although about 25% of the tripeptide repeats are either Gly-Pro-Y or Gly-X-Hpr, most other amino acids are found in X and Y positions. The individual peptide chains that form the triple helix are designated as α chains. Type I collagen, found mainly in skin, bone, and tendon, is comprised of two identical $\alpha1(I)$ chains and one similar $\alpha2(I)$ chain. Conversely, type II collagen, found in cartilage, and type III collagen, generally isolated from fetal calfskin and found together with type I collagen in mature skin, are both homotrimers comprised of three identical $\alpha1(II)$ and $\alpha1(III)$ chains, respectively.

The helical domain of bovine type I collagen is 1014 residues long. Each chain forms a left-handed helix of the polyproline II type, having three residues per turn with a pitch of 0.94 nm. The Gly residue in every third position allows these helices to pack together in the formation of a right-handed triple helix. In this structure, the three chains are staggered by one residue, with the Gly residues forming a shallow helix in the center and the X and Y sidechains directed outward to form a helical ridge.[21,23] Tropocollagen, the triple helical collagen monomer, is stabilized by peptide and hydrogen bonds as well as steric interactions. Gly residues permit close packing; rigid Pro rings prevent rotation about the peptide bond, while the hydroxyl groups of Hpr and functional sidechains of other amino acids provide sites for water bridges and hydrogen bonds as well as electrostatic and hydrophobic sidechain interactions.

Self-association of individual triple helices leads to the formation of supramolecular structures classified as microfibrils, fibrils and fibril bundles or fibers in increasing order of size and complexity. Many of the details of collagen ultrastructure (fibrils and fibers) were obtained by X-ray diffraction and electron microscopy. Although no microfibrils were isolated, a variety of models were proposed. These models were developed from cross sectional views of collagenous materials and generally considered four to six helices as the primary unit of the microfibril. Because the models were designed to explain experimental data, the exact composition of the microfibril had little effect on the information gained concerning inter-helical spacing.

Recently, Orgel et al.[24] used synchrotron radiation to observe in situ microfibril structure, and determined that interdigitation with neighboring microfibrils prevented the isolation of a single microfibril.

When viewed through a microscope, collagenous materials exhibit a periodic banding pattern characteristic of the collagen fiber. The origin of this pattern is the quarter-stagger arrangement of individual molecules, resulting in gap and overlap regions. For type I collagen, the helix is a 1014 residue long segment having the -Gly-X-Y- sequence, with short segments, not Gly-X-Y, called telopeptides, at the amino and carboxyl ends. The repeating unit of the banding pattern is called a D space, and a molecule is 4.4 D space long with 0.6 D gap; the assembly is a flexible rod about 1.5 nm by 300 nm.[23]

In the 1950's, X-ray diffraction patterns of collagen showing a highly organized supramolecular structure led observers to conclude that the structure was helical, but not α-helical. At about the same time, knowledge of the unique features (33% glycine, high levels of imino acids, and post translational hydroxylation of prolines and lysines) of the primary structure became available and encouraged early model builders to visualize the triple helix in ball and stick representations.[25] By the 1980's, computer-based molecular simulations had become feasible, and the Scheraga group at Cornell University explored the effects of various X and Y residues on the stability of the collagen triple helix.[26]

Leather Models
When I joined the ERRC leather research group in 1990, one of my assignments was to assist the industry in identifying an ecologically sound replacement for chromium in the production of fine leathers, more an issue of public perception than of real environmental concern. By this time, the industry had dealt with most of the air and water issues surrounding the production of leather, and my colleagues at ERRC were well into their research on the treatment of chromium-containing solid tannery waste.[27] With time, it became apparent that underlying the specific objective of finding an alternative to chromium was the need for a molecular level understanding of the mechanisms of tanning. Although different tannages produce leathers that are distinguishable, all are recognizable as leather and many of the steps in tanning are common to all.

As a scientist whose research could generally be described as protein structure/function relationships, I joined a research group that had already begun the development of a collagen molecular model comprehensive enough for the study of interactions (crosslinks) between different triple helical monomers. To complement the modeling work, I called upon the knowledge of my colleagues with greater experience in leather research and particularly the professional tanners,

employed as leather industry specialists at ERRC, in the development of laboratory scale model systems for observing, by spectroscopy, the effects of typical tannery processes on the structure of soluble collagen.

The ERRC leather research group was fortunate to have three excellent modelers as postdoctoral associates. In 1989, James Chen used the conformational parameters developed by the Scheraga group[26], the amino acid sequence of type I bovine collagen, and published X-ray diffraction data to begin the construction of a microfibril model. Because no single defined microfibril structure was available, he chose the 'Smith' model,[28] comprised of five triple helices in a left-handed coil for the ERRC model.[29] He used computer algorithms to first construct a $(Gly-Pro-Hpr)_{12}$ chain in the form of a left-handed helix, and then assemble three identical chains into an energy minimized right-handed coiled-coil to produce a triple helical template. Five of these triple helices were then supercoiled to form a left-handed Smith microfibril template.[29] Next, a sequence alignment template based on the Smith model was used to identify the amino acid side chains that would represent a 36 residue long slice of the microfibril. These side chains were mutated, computationally, into the positions originally occupied by Pro and Hpr side chains (Figure 1).[30,31]
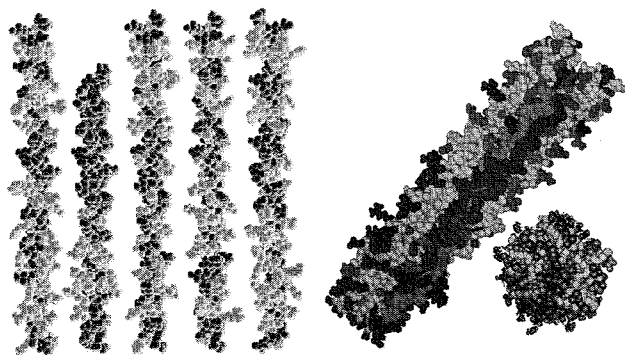

Figure 1: The original 36-residue long microfibril model, left to right as individual triple helices, lateral view of the assembled microfibril, and end on view of the microfibril.

When a similar path was later attempted by Gregory King in the construction of the full ERRC microfibril model, the direct substitution of actual amino acid residues for Pro and Hpr resulted in many bad contacts and general distortion of the backbone. King developed a procedure for growing new side chains in place, one bond at a time, to produce a model that when colored to represent a negative microscopy stain, showed a banding pattern typical of those seen in collagen micrographs (Figure 2).[32] This 15-chain model, encompasses a single gap region, 159 residues long, and has a 78-residue-long overlap region at either end. Because the individual chains are staggered, the entire tropocollagen sequence is represented.[33]
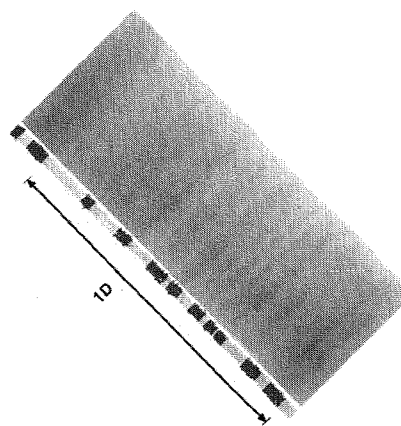

Figure 2: Computer model of the ERRC microfibril aligned with an electron micrograph of stained collagen fibers. In order to more easily compare the model to the micrograph, ionized sidegroups were colored to simulate microscopy staining.

Although the importance of telopeptides as anchors for the crosslinks that form in collagen as the animal ages was recognized, the initial model ignored these nonhelical regions because of the limited data then available on their secondary structures. In 1999, based on data for isolated telopeptides,[34,35] Phoebe Qi began the construction of models of the N-terminal[36] and C-terminal[37] telopeptides. These were fit into the gap region of the larger model and attached to the appropriate triple helices. The telopeptide conformations have since been modified in light of more recent studies of their interactions with the triple helical domain of collagen (Figure 3).[38,39]
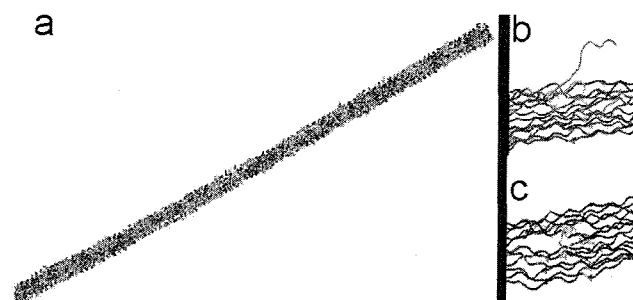

Figure 3: (a) The ERRC collagen microfibril model with enlarged views of the (b) N-terminal and (c) C-terminal gap-overlap interfaces with telopeptides.

The impetus for construction of this model was to provide a basis for the study of those interactions between tanning materials and collagen that might be expected to contribute to the stabilization of the fibril structure. Our first attempt was to explain the finding of Scholnick et al.[40] that although dicarboxylic acids with varying chain lengths bound to collagen, those with C-7 to C-12 chains had a much greater effect on collagen thermal stability than did either shorter or

longer chains. By simulating the acid molecules under experimental conditions, we were able to estimate the potential crosslinking span of different chains, and to show that short chain dicaboxylic acids would be able to crosslink within a triple helix, medium length chains could be expected to form inter helical crosslinks, and longer chain molecules might bind to the surface, but be unable to fit within the microfibril.

Most theories of chemical crosslinking or stabilization of collagen structure by tanning agents have focused on the triple helical domains of collagen because these were the regions best described and understood. One role of chromium in the tanning of leather is to crosslink the collagen fibrils by forming complexes with the carboxyl groups of glutamic and aspartic acid side chains on collagen.[41,42] Other mineral tannages were generally assumed to follow the chromium model. Vegetable tannins were thought to react with collagen by means of electrostatic, hydrophobic, and coordinate forces[41] and to crosslink or cluster between basic sidechains. Glutaraldehyde was considered to interact with collagen through lysine-to-lysine crosslinks via either the monomer or a condensed polymer as the tanning agent.[43] Acrylamide derivatives such as N,N'-methylenebisacrylamide could form crosslinks between lysine sidechains.[44] Thus, our next effort, based on the assumption that some aspects of tanning involve crosslinks anchored by either an acidic or a basic side chain on collagen, was a three dimensional analysis of the relative locations of ionizable groups,[45] based in part on the earlier two dimensional analysis of collagen primary structure.[46]

Although the primary chrome-tanning agent, "basic chrome sulfate," had been characterized as a mixture of Cr(III) complexes, where two or more chromium atoms are connected by oxygen and/or hydroxyl bridges with varying numbers of associated sulfate ions,[47] our expertise and the modeling software available were protein-based, and could not easily accommodate complex inorganic structures. Our rather primitive attempts at modeling chrome tanning were restricted to identifying potential binding sites with suitably spaced acidic residues.[42] In any event, more recent studies[48-50] suggest that tanning may better be described in terms of protein modification than as simple crosslinking.

A greater awareness of collagen modification and fibril coating in tanning has recently increased interest in tanning agents other than chromium, and a better understanding of the role of telopeptides in fibril stabilization has led to a closer examination of the gap region. A successful tanning agent is one that interacts with the collagen matrix of the hide in a way that provides stability. Under the conditions of tanning, most tanning agents are in oligomeric form and effective interactions with collagen are intermolecular. Thus one of the requirements for tanning may be adequate open space within the fiber structure to accommodate a moderately sized oligomer without major distortion of the collagen ultrastructure. We chose catechin, a polyphenolic vegetable tannin, for computer simulation to evaluate potential collagen-tannin interactions.[51] This model was used to estimate the accessibility of potential interaction sites in the microfibril structure, and to suggest the most likely types of interactions between tannin and collagen after simulated tanning (Figure 4). As would be expected, the atomic density around potential binding sites was lower in the gap region than in the overlap region. Intra- and interhelical hydrogen bonding and hydrophobic interactions were observed with this model. We have now begun to look at larger tannin molecules to see how they might best fit within a collagen fiber.
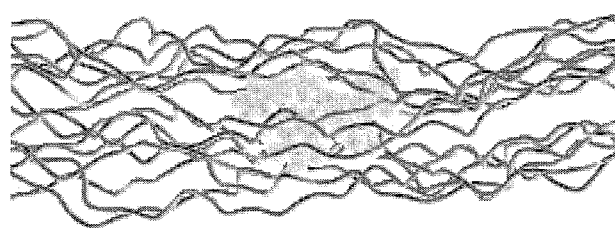

Figure 4: Catchin molecule placed within the gap region of the microfibril.

Although computer-modeling studies can be quite valuable in predicting or explaining experimental observations, and they may suggest which experiment among several is most likely to confirm or refute a hypothesis, the experimental test of the hypothesis is nevertheless essential. When working with a large insoluble substrate such as cattle hide, it is difficult to isolate the effects of individual processing steps on the molecular or supramolecular structure of collagen. Therefore, to complement our computer modeling studies, we have developed laboratory scale model-tanning systems.

For experimental observation of the effects of individual steps in a tanning process on collagen structure, we purchased pepsin-solubilized collagen and characterized it by polyacrylamide gel electrophoresis, and ultraviolet and circular dichroism spectroscopy to establish that it had the native characteristics and thermal stability of triple helical collagen. We then developed a very simplified model using conditions that approximated some steps in tanning processes[52] to explore the interactions of a chrome(III) sulfate complex[53] with collagen in solution. With this model, measurable effects on the conformation and conformational stability of soluble collagen under the conditions of chrome tanning could be seen. Partial denaturation of the helix appeared to be an essential step before chromium binding could occur, and the partially denatured conformation was stabilized in a chromium-collagen complex that remained soluble at the concentration used in this study. When

aluminum sulfate was substituted, to avoid the color associated with chrome salts, similar effects were more readily seen.[54] This model was also used to investigate the effects of added sodium and potassium salts[55,56] on the thermal stability of the collagen triple helix. Those studies suggested that salt levels typical of tanning processes would have water-structuring effects around the collagen molecules.

A powdered hide model was also developed for investigating the conditions necessary for tanning with natural tannins and other plant-based materials. One tanning material we have examined extensively is genipin, an iridoid compound isolated from the fruit of *Gardenia jasmindides* Ellis. Its properties as a tannage for bovine powdered hide, alone[57] and in combination with aluminum, glutaraldehyde, and vegetable tannins,[58] have been evaluated. The effects of variations in the parameters, pH and temperature of reaction, on the resulting thermal stability are now available for use with the computer modeling system to investigate the genipin-collagen mechanism.

Biomaterial Models
For biomedical and other industrial applications of collagen, the protein is usually isolated from the skin, tendon or bone and then modified or crosslinked to form a stable matrix not unlike that of leather. Researchers with a variety of interests have developed models and occasionally synthesized collagen peptides that could be crystallized to examine specific aspects of collagen structure. The perturbation of the triple helical structure of collagen peptides by benign or pathological mutations in the primary structure has been an active area for research, reviewed recently by Brodsky et al.[59] On a different scale is the work of Buehler[60] to model the mechanical characteristics of collagen fibers. Several groups have explored the effects of hydration on the stability of collagen peptides.[61-63]

The unique characteristics of the fiber-forming collagens make them useful ingredients for the manufacture of biomaterials. The dimensional stability of collagen is closely related to its organized molecular structure and results from the interplay of electrostatic, hydrophobic, and van der Waals interactions in addition to hydrogen and covalent bonds. The hydrothermal stability of a manufactured biomaterial such as leather will reflect the stability of collagen structures at the molecular level as well as at several levels of supramolecular structure. Although the transition temperatures characteristic of the hydrothermal stability of collagen in solution or in a fibrous matrix will vary with the level of supramolecular structure in the substrate, ultimately the characteristics of the particular collagen will determine those of the product. Thus hydrothermal stability is an important factor in the evaluation of collagen-based materials. Calfskin is a common source of fibrous collagen prepared in research laboratories.[64] On the other hand, commercially available collagens are commonly prepared from the skin or tendons of mature animals and thus the latter may be better models for biomaterial studies.

For an experimental biomaterial model, the Collagen Research Group at Widener University supplied us with insoluble collagen from the dermal layer of bovine hide. A dilute aqueous suspension of crude collagen was ball-milled for seven days to produce a loose collection of separated fibrils that is the starting material for several environmental and biotechnology applications.[65] The active surface area, of the milled collagen suspension, defined as that surface area able to interact with the liquid in which the collagen is blended, was estimated by scanning electron microscopy to be 0.01 to 0.3 m^2g^{-1}. This large active surface area is key to producing high viscosity collagen dispersions at very low concentrations. Somewhat surprisingly, the ball milling had no discernable effect on the collagen, except for the separation of fiber bundles.[66] Collagen dispersions useful in a variety of biotechnology applications such as artificial organ scaffolding, skin grafts and cell immobilization beads for fluidized bed reactors are generally stabilized by crosslinking to add strength and protect against enzymatic degradation. We evaluated glutaraldehyde and microbial transglutaminase crosslinking, and dehydrothermal drying as processes for stabilizing these collagen fibrils, and assessed their effectiveness by determining free amine residues remaining after cross-linking, collagenase resistance and size of cross-linked complexes. Glutaraldehyde cross-linking resulted in the least number of free amines, highest molecular weight aggregates and highest resistance to collagenase degradation. Dehydrothermal drying also produced high molecular weight aggregates, but the protein was partially denatured, making the fibril more susceptible to collagenase. Microbial transglutaminase-treated collagen contained large molecular weight aggregates and was more resistant to collagenase degradation than was control collagen.[67]

Numerous research groups worldwide are working with collagen and gelatin to learn the mechanisms of collagenous tissue organization. The objective generally is to enable the design of materials for use in organ replacements, scaffolds for cell growth, and other biomaterials. Research into the characterization and uses of reconstituted collagen fibers has a long history.[68] Recent attention has focused on the assembly of collagen fibers and microfibrils on surfaces that act as templates.[69-71]

Mineralized collagen as the basis for bone replacement scaffolds is an active area of biomedical research. Bone naturally assembles via a complex hierarchical nano-fibril system that has now been approximated in the laboratory by growing hydroxyapatite crystals on the surface of self-assembling collagen fibrils.[72] In a second approach to the

production of bone-like material, gelatin with ammonium phosphate and calcium nitrate was used to prepare precursor crystals for hierarchical assembly.[73] For regeneration of hard tissue that is not intended to be load-bearing, nanofibrous biocomposite scaffolds of type I collagen and nanohydroxyapatite were prepared by electrostatic cospinning. These scaffolds have a porous nanofibrous morphology with random fibers in the range of 500-700 nm diameters and a tensile strength three times that of unmodified collagen.[74]

As a basis for soft tissue engineering studies, type I collagen gels are biocompatible and biodegradable, both advantages, but have limited mechanical properties and thermal stability.[75] Current research exploring the roles of other extracellular matrix components in collagen organization shows that addition of either type III collagen or chondroitin sulfate results in a decreased fibril diameter and a gel with a more open structural network that can be useful in tailoring biomaterials for tissue engineering.[76] Cross-linking of collagen with gold nanoparticles via carbodiimide coupling significantly reduced the pore size in collagen gels and increased collagenase resistance. A cell viability assay indicated that the gold nanoparticles were not toxic at the concentrations used in gel synthesis; thus this new material has potential for the delivery of small molecule drugs as well as for photothermal therapies, imaging, and cell targeting.[77]

Blends of collagen and glycosaminoglycan, native constituents of mammalian tissues, are widely utilized to fabricate scaffolds that mimic the structure and biological function of the extracellular matrix, and may potentially be useful in soft tissue engineering applications. Novel nanofibrous scaffolds produced by electrospinning collagen with chondroitin sulfate (CS) exhibited a uniform fiber structure in the nano-scale diameter range. When crosslinked with glutaraldehyde vapor, these scaffolds had enhanced biostability and resistance to collagenase, and promoted fibroblast proliferation. Incorporation of CS into collagen nanofibers without crosslinking did not increase the biostability but still promoted cell growth.[78] Electrospinning is a fabrication process that uses an electric field to control the deposition of polymer fibers to fabricate mats composed of fiber diameters ranging down to 100 nm or less. Conditions optimized for calfskin type I collagen produce a matrix of 100 nm fibers that exhibit the 67 nm banding pattern characteristic of native collagen. Electrospinning is an efficient process that can be used to selectively deposit polymers in a predetermined pattern, making it possible to fabricate complex, and seamless, three-dimensional shapes. The structural, material, and biological properties of electrospun collagen suggest that this material may represent a nearly ideal tissue engineering scaffold.[79]

CONCLUSIONS

Covalent crosslinks, electrostatic interactions (salt bridges), hydrophobic interactions, hydrogen bonds, and water activity are among the contributing factors in a stabilization mechanism. Molecular modeling is an excellent approach to the study of the effects of these factors, individually and collectively, on collagen structure. A recognized limitation of the ERRC collagen modeling studies is that limited resources have made it impractical to perform the kinds of studies with the microfibril model that would be most useful to an understanding of tanning mechanisms. Other researchers with different resources have accepted the challenge of tanning-relevant modeling. At the 1997 IULTCS meeting, Jens Fennen, of TFL, reported on studies of two 36 residue triple helices in water and with a binuclear chromium complex as a potential tanning agent,[80] and Robert Docherty, of Stahl, reported the results of his preparation for solvation studies on a collagen triple helix.[81] Since 2004, a modeling group at the University of Pisa, Italy, has used the ERRC microfibril model as a basis for studies of the structural and binding properties of supramolecular collagen, as well as the adsorption of collagen on titanium surfaces.[82-84] They have also reported models for a vegetable tannin[85] and a chromium-polyphenol complex.[86] In 2006 and 2007, Lorenz Siggel, of BASF, reported leather-related studies on modeling of the collagen fibril in water and the effects of pickling and retanning agents.[87-89]

Research is most useful as a collaborative enterprise, or at least one where there is feedback between competing teams. The ERRC modeling group developed a collagen model suitable for studies of tanning mechanisms that has served as a catalyst for the initiation of those studies by others. The feedback from other researchers has enabled us to validate and strengthen the model. We continue to use our model to explore collagen /tanning agent interactions, particularly in the less dense gap region of the microfibril, and perhaps encourage others to adopt this focus. The requirement for experimental models as an adjunct to computer models is recognized, as is the necessity to be aware of and utilize appropriate models developed by scientists with different goals. Research on collagen as a biomaterial is a rapidly growing field and one expected to develop knowledge valuable for the tanner.

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